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(54) **Improved formulation for recombinant beta-interferon processes for recovery and stabilization of beta-interferon and the use thereof.**

(57) An improved process for recovering and purifying recombinant beta-interferon from its host yields a beta-interferon preparation which may be formulated into a stable pharmaceutical composition having a therapeutically effective amount of the biologically active recombinant beta-interferon dissolved in a non-toxic, inert, therapeutically compatible aqueous-based carrier medium at a pH of 2 to 4 which medium also contains a stabilizer for the recombinant beta-interferon, such as human serum albumin, normal serum albumin and human plasma protein fraction.

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IMPROVED FORMULATIONS FOR RECOMBINANT BETA-INTERFERON PROCESSES FOR RECOVERY AND STABILIZATION OF BETA-INFERON AND THE USE THEREOF

This invention is in the general field of biochemical engineering. More specifically, the subject invention relates to the preparation of biologically active recombinant lipophilic proteins such as human interferons. Still more specifically, the subject invention relates to an improved process for the production and recovery of lipophilic proteins from genetically transformed host organisms, lipophilic protein preparations of relatively high purity, and therapeutically acceptable formulations thereof.

Naturally occurring interferons (IFNs) are species-specific proteins, often glycoproteins, produced by various cells upon induction with viruses, double stranded RNA's, other polynucleotides, antigens and mitogens. Interferons exhibit multiple biological activities such as antiviral, antiproliferative, immunomodulatory and anticellular functions. At least three distinct types of human interferons have been identified and characterized in terms of their anti-viral, anti-growth and activation of natural killer cell (NK) activities. They are produced by leukocytes, lymphocytes, fibroblasts and the immune system and are classified as α, β and γ interferons. These are reported to be different proteins coded for by distinct structural genes.

Native human β-interferon (β-HIFN) is generally produced by superinducing human fibroblast cultures with poly-IC (polyribоinosinic acid and polyribocytidylic acid) and isolating and purifying the β-HIFN thus produced by chromatographic and electrophoretic techniques. Proteins or polypeptides which exhibit native β-interferon like properties may also be produced using recombinant DNA technology by extracting poly-A-rich 12S messenger RNA

from virally induced human cells, synthesizing double-stranded c-DNA using the m-RNA as a template, introducing the c-DNA into an appropriate cloning vector, transforming suitable microorganisms with the vector, harvesting the bacteria and extracting the β-HIFN therefrom. Nagola, S. et al., Nature, 284:316 (1980); Goeddel, D. V. et al., Nature, 287:411 (1980); Yelverton, E. et al., Nuc. Acid Res., 9:731 (1981); Streuli, M. et al., Proc. Nat'l. Acad. Sci. (U.S.), 78:2848 (1981); European Pat. Application Numbers 28033, published May 6, 1981; 321134, published July 15, 1981; 34307 published August 26, 1981; and Belgian Patent 837397, issued June 1, 1981 describe various currently used methods for the production of β-interferon employing recombinant DNA techniques. The expressed proteins or polypeptides have been purified and tested and have been found to exhibit properties similar to those of native IFNs. Bacterially produced IFN's thus appear to have potential therapeutic use as antiviral and anti-tumor agents and the production of IFN's by such bacterial fermentations is expected to yield sufficiently large quantities of IFN at a relatively low cost for clinical testing.

Protein samples for use in clinical studies, however, must be of relatively high purity and substantially uncontaminated with toxic host cell constituents, cell debris and other extraneous chemicals introduced during the extraction and purification steps. There are several methods currently available for the preparation, recovery and purification of bacterially produced proteins.

U.S. Patent No, 4,315,852 to Leibowitz et al. describes and claims a method for the acid extraction of leukocyte interferon from bacterial cells and neutralization of the extractant to obtain the interferon.

Derynck et al., Nature, 287:193 (1980) teach lysing transformed E. coli cells using a solution containing 5 M urea, 1% sodium dodecyl sulfate (SDS), and 1% 2-mercaptoethanol. The lysate, which was purified by chromatography, exhibited interferon activity.

Scandella and Kornberg, Biochemistry, 10:4447 (1971) describe the preparation of a phospholipase from E. coli by

solubilizing the cell membranes with SDS and precipitating the solubilized protein with 1-butanol.

U.S. Patent No. 4,343,735, to Menge et al. teaches a process for the purification of interferon by partitioning it in an aqueous multi-phase system in the presence of ion exchangers which are soluble in the system and are derivatives of polyethers.

U.S. Patent No. 4,343,736 to Uemura et al. discloses a method for recovering interferon by absorption on water-insolubilized heparin and then eluting the interferon with an aqueous solution of an inorganic salt and chondroitin sulfate.

U.S. Patent No. 4,289,689 to Friesen et al. discloses how to recover and purify human native β-interferon by use of affinity chromatography and high pressure liquid chromatography.

U.S. Patent No, 4,460,574 to Yabrov discloses a pharmaceutical composition comprising native human α- and β-interferons used for rectal or urogenital treatment of human interferon-sensitive diseases.

U.S. Patent No. 4,364,863 to Leibowitz et al. describes a method of extracting fibroblast interferon from bacteria using a low pH followed by a high pH extraction procedure.

PCT WO 80/02229 to Benzon discloses purification of alpha (leukocyte) interferon, which is not a lipophilic protein.

EP 42,246 discloses that recombinant interferons may be dissolved in any pharmaceutically acceptable non-toxic carrier appropriate for the desired form of administration without further details.

U.S. Patent No. 4,450,103 discloses solubilizing the protein in an aqueous medium with an appropriate solubilizing agent, extracting the protein from the aqueous medium with 2-butanol or 2-methyl-2-butanol, and precipitating the protein from the alcohol phase.

Cancer Treatment Reports, 62, 1900-1906 (1978) and EP 89,245 disclose that native beta-interferon may be formulated directly with human serum albumin in a pharmaceutically compatible aqueous-based medium at a pH of 7.2-7.8.

Alpha-interferons and native beta-interferon are not lipophilic proteins. Therefore, they can be stabilized and solubilized by adding a stabilizer such as human serum albumin directly to the formulation at physiological pH. In contrast, lipophilic proteins such as recombinant beta-interferon are not solubilized by addition of human serum albumin at pH 6.8-7.8.

A major problem with the existing methods of purification and recovery of lipophilic proteins is that the protein is not produced in a sufficiently pure form and in sufficiently large quantities for clinical and therapeutic purposes, and further, that the resulting protein preparations, especially those that are produced by recombinant DNA techniques, have residual toxic amounts of chemicals, such as SDS and other surfactants or precipitants used in the extraction and purification steps. Thus, these preparations are not acceptable for clinical studies designed to determine the extent of the therapeutic use and applications of these proteins. It would be desirable, therefore, to have available a process for the recovery of a lipophilic protein in sufficiently large quantities and without toxic levels of SDS for clinical and therapeutic applications.

EP 158,487, published October 15, 1985, discloses an interleukin-2 composition which comprises human serum albumin, a reducing compound or a combination thereof, and which is adjusted to a pH from 3 to 6 as a solution.

The present invention enables the provision of pharmaceutically acceptable samples of recombinant β-interferc which is of relatively high purity, ideally in sufficiently large quantities for clinical and therapeutic applications.

The instant invention in other aspects aims to enable the provision of recombinant β-interferon preparations which are substantially free of SDS, e.g. wherein the level of SDS is less than about 10 p.p.m., ideally without loss of their biological activity, or at levels that are therapeutically acceptable.

U.S. Patent No. 4,462,940 describes an improved method for the production, recovery and purification of a lipophilic protein such as human recombinant β-interferon which comprises solubilizing the protein into an aqueous medium with a suitable solubilizing agent, extracting the solubilized protein with an aliphatic alcohol, precipitating the protein from the alcohol phase with an aqueous buffer, and diafiltering the protein at a pH of about 10.5 to 12.5, preferably at a pH of about 12, against water adjusted to a pH of about 10.5 to 12.5, preferably about 12, or against mixtures of water and aliphatic alcohols, preferably ethanol and glycerol adjusted to a pH of about 10.5 to 12.5, preferably about 12, substantially to remove SDS or to reduce its concentration to therapeutically acceptable levels. The protein sample is optionally purified by conventional methods such as chromatography prior to the diafiltration.

A preferred embodiment of the above method comprises recovering bacterially produced human β-interferon by disruption of the bacterial cells, solubilization of the interferon with a suitable solubilizing agent, extracting the solubilized interferon with an aliphatic alcohol of 2-6, preferably 4-6 carbon chain length, precipitating the interferon from the alcohol phase, further purifying the interferon by conventional methods, preferably gel filtration chromatography, and diafiltering the interferon fraction at a pH of about 10.5 to 12.5, preferably at a pH of about 11, against pure water or mixtures of water and aliphatic alcohols, preferably methanol, ethanol, propanol, butanol, glycerol and the like, also adjusted to a pH of about 10.5 to 12.5, preferably about 11.

The present invention concerns a stable pharmaceutical composition comprising a therapeutically effective amount of a biologically active recombinant beta-interferon dissolved in a non-toxic, inert, therapeutically compatible aqueous-based carrier medium at a pH of 2 to 4 comprising a stabilizer for the protein. Preferably, the stabilizer is human serum albumin, a mixture of human serum albumin and dextrose, human plasma protein fraction, or normal serum albumin.

The invention also concerns a process for recovering recombinant beta-interferon from a host transformed to produce it wherein the cell wall of the host is disrupted and the beta-interferon in the disruptate is isolated and purified, comprising the steps of:

(a) adjusting the pH of the medium in which the beta-interferon is contained to about 2 to 4;

(b) adding to the beta-interferon medium a stabilizer for the protein which has been previously adjusted to a pH of about 2 to 4; and

(c) lyophilizing the resulting composition at about pH 2 to 4.

Once the pH has been adjusted to a pH of about 2 to 4, it can be raised to a pH range of preferably 6.8-7.8.

The process can further comprise the step of adding a non-toxic, inert, therapeutically compatible aqueous-based carrier to the composition.

Figure 1 illustrates a flow chart of the process steps of the present invention wherein the pH is raised to alkaline pH during diafiltration.

Figure 2 shows a graph plotting percentage of the total recovery of recombinant human β-interferon and purity of the product during the precipitation step, as a function of pH in the range of about 4-8.

Figure 3 is a representation of a chromatographic chart illustrating the homogeneity of the recombinant human β-interferon fraction eluted after three passes through a vinyl polymer gel column.

Figure 4 is a plot of the antiviral activity of recombinant human β-interferon as a function of pH in the range of about 6-12.

Figures 5a and 5b sequentially illustrate a flow chart of the process steps of the present invention wherein the protein is recovered from refractile bodies and the pH is lowered to acidic pH for stabilizer addition.

Figure 6 illustrates a flow chart of the process steps of the present invention wherein the protein is extracted and solubilized and the pH is lowered to acidic pH for stabilizer addition.

As used herein, the term "lipophilic protein" refers to a protein which is not soluble or not readily soluble in an aqueous medium under ambient conditions of room temperature and atmospheric pressure at a pH of between about 6.5 and 7.8. Examples of such proteins include human recombinant β-interferon and immunotoxins prepared by conjugating a cytotoxin moiety such as ricin A to an antibody against a pathologic condition such as breast cancer. The term "recombinant protein" refers to a protein which is produced by recombinant DNA techniques wherein generally DNA is inserted into a suitable expression plasmid which is inserted into a host organism not native to the DNA which is transformed to produce the heterologous protein. The host may be any organism foreign to the DNA such as, e.g., bacteria, yeast, viruses, mammals, etc. Preferably the host is microbial, and most preferably is bacterial.

As used herein, the term "β-HIFN" refers to human β-interferon or β-interferon-like polypeptides produced by recombinant DNA techniques and whose amino acid sequence is the same as or similar or substantially homologous to the unglycosylated and/or glycosylated native β-interferon.

The precise chemical structure of the protein will depend on a number of factors. As ionizable amino and carboxyl groups are present in the molecule, a particular protein may be obtained as an acidic or basic salt, or in neutral form. All such preparations which retain their activity when placed in suitable environmental conditions are included in the definition of proteins herein. Further, the primary amino acid sequence of the protein may be augmented by derivatization using sugar moieties (glycosylation) or by other supplementary molecules such as lipids, phosphate, acetyl groups and the like, more commonly by conjugation with saccharides. Certain aspects of such augmentation are accomplished through post-translational processing systems of the producing host; other such modifications may be introduced in vitro. In any event, such modifications are included in the definition of protein herein so long as the activity of the protein, as defined above, is not destroyed. It is expected, of course, that such modifications may quantitatively or qualitatively affect the activity, either by enhancing or diminishing the activity of the protein in the various assays.

Further, individual amino acid residues in the chain may be modified by oxidation, reduction, or other derivatization, and the protein may be cleaved to obtain fragments which retain activity. Such alterations which do not destroy activity do not remove the protein sequence from the definition.

Finally, modifications to the primary structure itself by deletion, addition, or alteration of the amino acids incorporated into the sequence during translation can be made without destroying the activity of the protein. For example, at least one cysteine residue which is not essential to biological activity, is present in the biologically active protein, and is free to form a disulfide link may

be deleted or replaced with another amino acid to eliminate sites for intermolecular crosslinking or incorrect intramolecular disulfide bond formation. Such modified proteins, known as "muteins," are described in U.S. Patent No. 4,518,584 issued May 21, 1985. In another example, a conservative amino acid of a biologically active protein such as IFN-β is substituted for each methionine residue susceptible to chloramine T or peroxide oxidation, wherein additional, non-susceptible methionine residues are not so substituted. A conservative amino acid alteration in this context is defined as one which does not adversely affect biological activity and involves neutral or non-polar amino acid substitutions or deletion of the methionine.

The protein of greatest interest herein is a β-HIFN. Most preferably the protein is an unglycosylated β-HIFN which is produced by a microorganism that has been transformed with a human IFN-β gene or a modification of the human IFN-β gene that encodes a protein having: (a) an amino acid sequence that is at least substantially identical to the amino acid sequence of native human IFN-β and (b) biological activity that is common to native human IFN-β. Substantial identity of amino acid sequences means the sequences are identical or differ by one or more amino acid alterations (deletions, additions, substitutions) that do not cause an adverse functional dissimilarity between the synthetic protein and the native human IFN-β. Examples of such proteins are the IFN-β proteins described in U.S. Patent No. 4,518,584. Most preferably, the IFN-β is ser_{17}IFN-β wherein the cysteine residue at amino acid position 17 is replaced by a serine residue.

As used herein, the term "physiological pH" refers to a pH which is pharmaceutically acceptable to mammals, i.e., a pH of about 7.2-7.6.

As used herein, the term "stabilizer" as applying to the lipophilic protein refers to non-toxic, non-therapeutic, non-immunogenic compositions which act not only to stabilize the diafiltered protein against denaturation and loss of biological

activity, but also to solubilize the lipophilic protein in an aqueous medium so that the pharmaceutical formulation constitutes an aqueous solution of diafiltered protein at pH 6.8-7.8 from which the protein will not precipitate. Such stabilizers are not known in the art for their solubilizing function. Examples of such stabilizers include, but are not limited to, proteins or carbohydrates preferably chosen from the proteins human serum albumin (HSA), and human plasma protein fraction (PPF), and the carbohydrates mannitol, sorbitol, glycerol, dextrose or a mixture thereof.

The type of stabilizer employed and the concentration thereof will depend mainly on the pH method and formulation employed and on the protein. For example, for low pH formulations using IFN-β_{ser17}, PPF is preferred. PPF is commercially available and is composed of at least 83% albumin and no more than 17% globulins (α and β); no more than 1% of the proteins are gamma-globulins. The α- and β-globulins in blood plasma serve several functions, one of which is to hold in stable aqueous solution relatively insoluble blood components, including cholesterol, fat-soluble vitamins, and hormones. Carbohydrate stabilizers can only be used in formulations maintained/lyophilized at pH 2-4.

The final concentration of stabilizer generally may range from 0.1-10% (w/v) depending mainly on the protein and type of stabilizer and the pH employed, with higher ranges preferred for low pH. A range of 0.5 to 10% (w/v) is typical for HSA with β-HIFN; and 0.1 to 5% (w/v) is typical for PPF with β-HIFN.

Many of the methods used for the recovery of lipophilic recombinant proteins, such as bacterially produced β-HIFN, utilize SDS or similar surfactants for the solubilization and isolation of the protein from cellular material and subsequent acid precipitation to obtain the protein. By further purification techniques carried out at or near neutral pH, the SDS levels in the final protein preparations are reduced to about 0.1%, but even these residual levels have been found to be toxic in animal studies and thus not acceptable for therapeutic or clinical applications. Further removal of SDS by

diafiltration techniques in the 4-8 pH range results in almost complete loss of β-HIFN activity due to aggregation and precipitation of the protein. The biological activity of β-HIFN lost during diafiltration may be regained by the addition of SDS.

For a free or unbound solute, the removal rate during diafiltration from a well mixed vessel follows first order kinetics. Since unbound SDS below its critical micelle concentration is a molecule small enough to pass unhindered through a 10,000 dalton cut-off membrane, its removal rate would be expected to follow first order kinetics, and if that were the case, SDS at an initial concentration of about 1000 µg/ml should be reduced to less than 1 µg/ml after about seven volume replacements. It was found, however, that SDS removal from β-HIFN did not fit this theoretical model, indicating that there were protein-SDS interactions which significantly affected the kinetics of SDS removal, and that SDS removal from this bound state in the 4-8 pH range promoted protein-protein interactions resulting in aggregation or precipitation of the protein. Higher or lower pH ranges for SDS removal would not be expected to be desirable, as at higher or lower pH, some proteins are known to be denatured. However, according to the present invention, following the removal of SDS by diafiltration at low ionic strength or by desalting, increasing or decreasing the pH by the addition of base or acid, respectively, solubilizes the protein and essentially restores its biological activity in the formulation.

Thus, the present invention solves the problem of lipophilic protein aggregation and precipitation and loss of protein activity with the removal of SDS. One solution to the problem described in U.S. Patent No. 4,462,940 involves initially adjusting the pH to about 10.5 to 12.5 and diafiltering against distilled water or aqueous mixtures of alcohols, using a 10,000 molecular weight cut-off ultrafiltration membrane after optionally reducing a partially purified sample of the protein with dithiothreitol (DTT) or mercaptoethanol or glutathione or cysteine at about 60°C and a pH of about 8.5 to prevent aggregation of the protein. Exemplary alcohols include ethanol, butanols, glycerol, mannitol, sorbitol, dextrose and the like.

The instantly claimed solution to the problem involves initially adjusting the pH of the purified protein pool to about 2 to 4, adding the stabilizer previously adjusted to a pH of 2 to 4, optionally incubating the mixture and raising its pH to 6.8 to 7.8. Incubation time depends mainly on the type of protein, type of stabilizer, exact pH, and concentrations of protein and stabilizer, and typically ranges from 0-100 minutes, preferably 10-100 minutes, more preferably 15-60 minutes, and most preferably 15-45 minutes.

Further, in another but less preferred aspect, the instantly claimed solution to the problem is where the pH is low, the stabilizer and protein pool are mixed together and the pH of the mixture is adjusted to 2 to 4, and the pH is raised, gradually or at once, to 6.8-7.8.

The subject invention is therefore a process for the recovery of lipophilic recombinant proteins of relatively high purity which contain lower than toxic levels of SDS and which can be reconstituted into therapeutically acceptable formulations in an appropriate carrier medium, and lipophilic protein compositions, most preferably β-HIFN compositions, wherein SDS levels are at less than 10 p.p.m., usually in the range of 2-6 p.p.m.

For purposes of practicing the present invention, bacteria are the preferred microorganism hosts, with E. coli being the most preferred.

Generally, proteins are susceptible to denaturation, peptide bond hydrolysis, hydrolysis of individual amino acids, β-elimination, racemization, formation of different amino acids and similar reactions in the high alkaline pH range; but with β-HIFN, none of the degradative reactions enumerated above are detected. On the other hand, when the protein is diafiltered at a pH of about 11, the resulting β-HIFN is pure and homogeneous and exhibits high specific activity, close to that of native β-HIFN.

In a preferred embodiment of the process of the invention, the disrupted cells are treated to isolate and purify the β-HIFN protein, and then the following steps are carried out:

(r) desalting the protein by G25 chromatography at pH 9.2-11;

(s) adjusting the pH of the desalted pool to about 3.5;

(t) adjusting the pH of a solution of human serum albumin or plasma protein fraction to pH 3.5;

(u) adding the human serum albumin or plasma protein fraction to the desalted pool and incubating for 15-45 minutes;

(v) lyophilizing the protein sample, if desired; and

(w) reconstituting the lyophilized protein sample, if desired.

In another embodiment of the process of this invention, the disrupted cells are treated to isolate and purify the β-HIFN protein and then the following steps are carried out:

(r) desalting the protein by G25 chromatography at pH 9.2-11;

(s) adding human serum albumin or plasma protein fraction to the desalted pool to form a mixture;

(t) lowering the pH of the mixture to 3-4;

(u) incubating the mixture for 15-45 minutes;

(v) lyophilizing the protein sample, if desired; and

(w) reconstituting the lyophilized protein sample, if desired.

The β-HIFN is preferably oxidized so that its cysteine residues are bridged to form cysteines, as described by U.S. Patent No. 4,530,787 to Shaked et al., using o-iodosobenzoic acid solution or by U.S. Patent No. 4,572,798 to Koths et al., entitled "Method For Promoting Disulfide Bond Formation In Recombinant Proteins", using copper chloride. The disclosure of such patents are incorporated herein by reference.

The detailed procedure for obtaining the protein follows.

The transformed microorganisms are grown in a suitable growth medium, typically to an optical density (OD) of at least about 10 at 680 nm, and preferably between about 50 and 100 at 680 nm. The composition of the growth medium will depend upon the particular microorganism involved. The aqueous growth medium contains compounds that fulfill the nutritional requirements of the microorganism of choice. Growth media will typically contain assimilable sources of carbon and nitrogen, energy sources, magnesium, potassium and sodium ions, and optionally amino acids and purine and pyrimidine bases. (Review of Medical Microbiology, Lange Medical Publications, 14th Ed. pp. 80-85 (1980).) Growth media for E. coli are well known in the art. Depending upon the particular solubilizing agent used in the invention process it may be desirable to minimize the amount of substances in the growth medium that may decrease the solubility of the solubilizing agent in water. For instance, potassium ions affect the solubility of SDS and, therefore, should be kept to a minimum when SDS is used as a solubilizing agent in the process, or removed by diafiltration following the concentration step.

Once the culture has reached the desired cell density, the cells are optionally killed or inactivated by heating or adding to the medium a cytotoxic agent such as chloroform or toluene that may be removed easily after the cells have been killed. The cells are thereafter optionally concentrated to about 20 to 150 mg/ml, preferably 80 to 100 mg/ml (OD 40 to 300, preferably 160 to 200 at 680 nm), by cross-flow filtration, centrifugation, or other conventional methods.

Following the concentration step, the cell membranes of the microorganisms are disrupted to facilitate the solubilization of the particulate matter in the concentrate. Protein assays for biological activity indicate that much of the protein is associated with (i.e., contained in or bound to) the cell membrane. Accordingly, disruption of the cell membrane enhances the contact of the solubilizing agent with the membranes and thus increases the rate at which the interferon associated with the membrane goes into solution. Conventional cell disruption techniques such as homogenization, sonication, or pressure

cycling may be used in this step of the process. A preferred method is using a bead mill or a pressure homogenizer. Either before or after the disruption, the pH of the liquid phase of the concentrate or disruptate, as the case may be, is adjusted, if necessary, to a level that facilitates dissolution of the solubilizing agent and the particulate matter in the concentrate/disruptate. The pH may be so adjusted by adding suitable buffers or with NaOH. In most instances a pH in the range of about 7 to about 8 is preferred.

Various techniques can be used to treat the disrupted cells. In one method, after the cells have been disrupted the particulate matter can be separated from the liquid phase of the disruptate and resuspended in an aqueous medium buffered to the optimal pH for solubilization. The protein concentration of the cell suspension after solubilization is in the range of about 2 to about 15 mg/ml, preferably 6 to 8 mg/ml.

The solubilization of the particulate cellular material, including the lipophilic recombinant protein, can be carried out concurrently with the disruption or sequentially following the disruption. It is preferably carried out as a separate step following the disruption. The solubilization is preferably carried to completion -- that is, substantially all of the particulate matter (e.g., protein, lipids, nucleic acids, phospholipids) in the disruptate is dissolved into the aqueous medium. Substantially complete dissolution of the particulate matter is achieved by adding an appropriate solubilizing agent to the aqueous suspension. Surfactants (detergents) that have a suitable hydrophobic-hydrophilic balance to solubilize the protein and which form a complex with the protein which can be extracted into the organic phase can be used in the invention. Strong natural or synthetic anionic surfactants such as alkali metal salts of fatty acids and alkali metal alkyl sulfates may be used. Such agents will usually contain 10 to 14 carbon atoms. SDS and sodium laurate are particularly preferred solubilizing agents. Examples of other solubilizing agents that can be used in the process include, but are not limited to, sodium dodecyl sulfonate, sodium decyl sulfate, sodium tetradecyl sulfate, sodium tridecyl

sulfonate, sodium myristate, sodium caproylate, sodium dodecyl N-sarcosinate, and sodium tetradecyl N-sarcosinate.

The amount of solubilizing agent used in the solubilization depends upon the particular agent and the amount of protein to be solubilized. In most instances, solubilizing agent to protein weight ratios in the range of about 1:1 to 10:1 are sufficient. When SDS is used, an SDS to protein ratio of about 1:1 to about 5:1, preferably about 3:1, is used. Temperatures in the range of 15°C to 60°C are generally used in the solubilization. Mixing may be employed to enhance contact between the solution and particulate matter and thus decrease the time it takes to dissolve the cellular matter. The solubilization is considered complete when the solution is substantially clear. Optical densities of about 4.0 to 8.0 at 280 nm are characteristic of the end point of the solubilization process.

Following the solubilization, the ionic strength of the solution is adjusted, if necessary, to a level at which the solution and organic extractant are substantially immiscible. The ionic strength is in the range of about 0.05 to 0.15. Inorganic salts, including NaCl and/or the like, are added to the solution for this purpose. Such ionic strengths enable phase separation after the extraction. The extractants used in the process are alcohols such as 2-butanol, 2-methyl-2-butanol, or mixtures thereof. The mixtures preferably contain less than about 50% by volume of 2-methyl-2-butanol. 2-Butanol is the preferred extractant. The ability of these alcohols to extract the lipophilic protein from the solubilizate is specific. The extractant is normally combined with the aqueous solution of the protein in volume ratios in the range of about 0.8:1 to about 3:1, preferably about 1:1 (extractant:aqueous solution). The extraction can be carried out using conventional batch or continuous liquid-liquid extraction techniques and equipment. The extraction is normally carried out at about 20°C to 100°C and involves contact times in the range of about one minute to one hour. The optimum contact time depends upon the particular solubilizing agent and extractant combination. When SDS is used, shorter times in the above range can be used. When sodium laurate is used, longer times in the range must

be used. The pH of the extraction mixture ranges between about 6 and 9, with a pH of about 7.5 being preferred when SDS is used, and a pH of about 8.5 when sodium laurate is used.

Upon completion of the extraction, the aqueous phase and extractant phase are separated and the protein is isolated from the extractant phase. The particular isolation procedure used depends upon the solubilizing agent involved and the desired degree of purity of the final product. Various isolation techniques such as precipitation, molecular sieve chromatography, affinity chromatography, and electrophoresis may be employed. In instances in which SDS is used, the desired lipophilic protein together with other proteins are precipitated from the extractant by mixing the extractant solution with aqueous buffer at volume ratios of about 2.0:1 to about 5:1, preferably about 3:1, and reducing the pH, typically to the range of about 5 to 7. The recovery of β-HIFN in the range of the pH 4 to 8, as shown in Fig. 2, shows a downward trend in the recovery of the protein with increasing pH, with an appreciable loss in the recovery of greater than 60% at a pH of about 8. Separation of the precipitate from the supernatant and evaporation of residual extractant from the precipitate provide a product that is greater than about 90% pure protein provided that the pH of the precipitation step is greater than 5.5. This product also contains minor amounts of nucleic acids (<1% to 2% by weight) and SDS (<1% w/v).

After further purification by methods known in the art, including but not limited to, chromatography, SDS may be removed in one embodiment of the invention by diafiltration at a pH of about 10.5 to 12.5, preferably about 12. The second purification step is optional and is not required for SDS removal by diafiltration. When sodium laurate is used as a solubilizing agent it precipitates from the extractant together with the protein on lowering the pH. Sodium laurate is extracted from the protein using organic solvents such as acetone, methanol and the like. Prior to diafiltration, the protein may be optionally reduced with appropriate reducing agents. Mercaptoethanol, glutathione, cysteine and dithiothreitol (DTT) may be employed for this purpose, with DTT being the most preferred.

The protein thus isolated is then solubilized in an aqueous-based carrier medium using a stabilizer as described above. The stabilizer, however, cannot merely be mixed with the protein for solubilization to occur. First the pH of the stabilizer must be raised to between 10.5 and 12.5 using a suitable base, preferably about 12, then the stabilizer is added to the diafiltered protein pool which is at pH 10.5-pH 12.5, and finally the pH of the resulting formulation is lowered to between about 6.8 and 7.8. Upon lowering the pH, the protein becomes solubilized in the medium.

Another method for treating the disrupted cells is shown in Figures 5a and 5b, which outline a process for recovering refractile bodies containing heterologous proteins from microbial hosts. In this method the protein is extracted from refractile bodies and solubilized with a denaturing agent such as SDS. The SDS is later removed by a desalting column. The pH of the eluate is adjusted to 2 to 4, the pH of a stabilizer is adjusted separately to pH 2 to 4, the stabilizer is added to the eluate, the mixture is optionally incubated for generally about 10-100 minutes, depending on the factors described above, and the pH is adjusted to between 6.8 and 7.8.

In yet another method, shown in Figure 6, the disruptate is extracted with a chaotropic agent, the protein is solubilized and reduced, and the reduced protein is separated, oxidized, purified and recovered. The formulation in Figure 6 is obtained using a low pH adjustment procedure with an incubation period.

The carrier medium used for the formulation for therapeutic or clinical administration may be any non-toxic, inert and aqueous-based vehicle such as those commonly used to formulate pharmaceuticals for animal or human administration. The carrier also is selected so that it does not affect the biological activity of the lipophilic protein.

Examples of such carriers include distilled water, physiological saline, Ringer's solution, dextrose solution, and Hank's solution. The same carriers can be used to reconstitute lyophilized lipophilic protein.

The present invention is further described by the following Example provided for purposes of illustration only and are not intended to limit the invention in any manner. In the Example all parts and percentages are by weight if solids and by volume if liquids, unless otherwise specified, and all temperatures are in degrees Celsius.

Example

IFN-β_{ser17} is a microbially produced mutein of IFN-β in which the cysteine residue at amino acid position 17 is replaced with a serine residue. IFN-β_{ser17} has two remaining cysteine residues: one at position 31 and the other at position 141. In native IFN-β the cysteines at positions 31 and 141 interact to form a disulfide bridge. The genetically engineered *E. coli* strain used in this example to produce IFN-β_{ser17} was deposited in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 USA on November 18, 1983 under accession number 39,517.

The genetically engineered *E. coli* mentioned above was grown in the following medium:

Ingredient	Approximate Initial Concentration
Na_3 Citrate · $2H_2O$	3 mM
H_2PO_4	30 mM
$(NH_4)_2SO_4$	74 mM
$MgSO_4$ · H_2O	3 mM
$MnSO_4$ · H_2O	46 µM
$ZnSO_4$ · $7H_2O$	46 µM
$CuSO_4$ · $5H_2O$	1-2 µM
L-tryptophan	350 µM
$FeSO_4$ · $7H_2O$	74 µM
thiamine · HCl	0.002% (w/v)
glucose	0.5% (w/v)

A 25% solution of Dow Corning Antifoam B, a 50% solution of glucose and 5N KOH were added on demand.

The temperature was maintained at 37 ± 1°C, the pH at 6.5 ± 0.1 with NaOH, and dissolved oxygen at 30% w/w of air saturation. Optical density and residual glucose measurements were taken at 14 hours and at approximately one-hour intervals thereafter. Harvest was made when glucose consumption reached 40 ± 6 g/l (OD at 680 nm = 10-11).

The harvested material was concentrated approximately 3-fold by circulating it through a microporous cross-flow filter under pressure. The concentrated cells were diafiltered against deionized water until the harvest material was concentrated 4-5 fold. The cells were then disrupted by passing them through a Manton-Gaulin homogenizer at 4.1-5.5 x 10^4 kpa (0.6-0.8 psi). After the initial pass sodium dodecyl sulfate (SDS)-sodium phosphate buffer was added to a final concentration of 2% w/v SDS, 0.08 M sodium phosphate, and solubilization was continued for one hour. Solid dithiothreitol (DTT)

was then added to a final concentration of 50 mM and the homogenate was heated to 90 ± 5°C for 10 minutes. The resulting cell suspension was extracted with 2-butanol at 1:1 2-butanol:suspension volume ratio in a static mixer. The mixture was then centrifuged and the 2-butanol-rich phase was collected.

The 2-butanol-rich phase was mixed with 2.5 volumes of 0.1% w/v SDS in phosphate buffered saline (PBS). Solid DTT was added to a final concentration of 1 mM. The pH of the mixture was adjusted to 6.2 ± 0.1 with glacial acetic acid and this mixture was centrifuged. The resulting paste was collected and resuspended in a mixture of PBS and 10% w/v SDS with pH adjustment to 8.5 ± 0.1 using 1 N NaOH. Solid DTT was added to a final concentration of 100 mM and the suspension was heated to 90 ± 5°C for 10 minutes. The suspension was then cooled to about 25°C, the pH was adjusted to 5.5 ± 0.1 with glacial acetic acid, and the solution was filtered.

The solution was then applied to a Sephacryl S-200 pre-column with a buffer consisting of 1% SDS, 50 mM sodium acetate, 1 mM EDTA, pH 5.5. The fractions containing highest interferon activities were pooled and concentrated by ultrafiltration with a 10 kilodalton molecular weight cut-off.

The protein was oxidized to generate sulfhydryl bonds using the method of Shaked et al., supra. A 1 mM o-iodosobenzoic acid solution was prepared by mixing the acid in water, sonicating the mixture for about five minutes and then stirring and adding 2% NaOH slowly to obtain a final pH of 8.2 ± 0.2 (additional sonication may be used as an alternative to adding base).

A reaction buffer medium was prepared by dissolving $Na_4P_2O_7 \cdot 10H_2O$ in water to a concentration of 2 mM. The pH of this solution was adjusted to 9.0 by adding 10% acetic acid, SDS to 0.1%, and ethylenediaminetetraacetic acid (EDTA) to 1 mM and the o-iodosobenzoic acid solution to 15 μM were added to the solution.

The buffer medium was placed in a reaction vessel equipped with a magnetic stirrer and a pH electrode set at 9.0. The $IFN\text{-}_{ser17}$ preparation and the o-iodosobenzoic acid solutions were added to the

reaction mixture from holding vessels using peristaltic pumps that were calibrated to introduce equivalent mole ratios of the IFN and oxidizing agent. The pH of the reaction mixture was controlled at 9.0 by adding 0.25 M NaOH via a peristaltic pump at 5 ml/hr as needed. The IFN-β solution (5 mg/ml in 50 mM acetate buffer, pH 5.5) was added at a flow rate of 2 ml/hr (7.0 micromole/hr) for about five hours; the o-iodosobenzoic acid solution was added at 7 ml/hr (7 micromole/hr) over the same time period. The addition of the acid solution was continued thereafter to get a final excess of 10-15 micromolar. The reaction was followed by reverse phase HPLC and by assaying the residual thiol content of IFN-β_{ser17} by Ellman's assay. After 6.5 hours the reaction was terminated by adding 10% acetic acid to the reaction mixture to a pH of 5.5.

The product was then loaded on a Sephacryl-200 column using a buffer consisting of 0.1% SDS, 1 mM EDTA, and 50 mM sodium acetate at pH 5.5. The monomer peak from this column was pooled and loaded on a Sephadex G-75 column using a buffer consisting of 0.1% SDS, 1 mM EDTA, and 50 mM sodium acetate at pH 5.5.

I. PPF Formulation

A total of 1.3 mg/ml of IFN-β from the G-75 pool was loaded on a desalting G25 Sephadex column equilibrated with a pH 11 buffer. A total of 2.2% plasma protein fraction (PPF) was adjusted to pH 3 and added to 5.56 ml of the desalted IFN-β, containing 0.25 mg/ml IFN-β. PPF is derived from Cohn Fraction IV_1. PPF is similar to HSA with the exception that PPF has more α- and β- globulins. Fraction IV_1 has the highest amount of α and β globulins. The IFN-β and PPF mixture was incubated for about 45 minutes and then adjusted to pH 7.5.

II. PPF/HSA Formulations

A total of 30 ml of the G-75 IFN-β pool above was concentrated to 10.5 ml and the pH was adjusted to 11. The concentrate was desalted on a G25 Sephadex column fully equilibrated to pH 11. The IFN desalted pool was used in the following experiments:

A total of 3.33 ml of the desalted IFN-β having 0.25 mg IFN per ml was used in each experiment and the pH of each mixture was adjusted to 3 before incubation and adjusted to between 7.3 and 7.5 after incubation. The results are indicated in the table below.

Stabilizer	Amount of Stabilizer (%)	Incubation Time (min.)	Clarity at pH 7.3-7.5
HSA	2.5	45	slightly hazy
HSA	5.0	45	very clear
HSA	2.5	15	slightly hazy
HSA	5.0	15	very clear
PPF	2.5	45	very clear
PPF	2.5	15	very clear

The PPF formulations were found, when examined visually, to have the best clarity, with 5.0% HSA next best, followed by 2.5% HSA. When each formulation was lyophilized and reconstituted with water, the PPF formulations reconstituted more clearly than the HSA formulations. All lyophilized formulations had IFN-β activity.

Experiments done at pH 3-4 without an incubation period appeared to show no appreciable difference from those that undergo an incubation period. Changes in concentration of PPF do result, however, in a marked difference in clarity.

Experiments to optimize the pH 3 formulation revealed that 5% HSA was distinctly better than the 2.5% HSA formulation. Also, increasing the incubation time from 15 minutes to 60 minutes assisted the solubility. The 5% HSA at 15 minutes incubation, however, was found to be better than the 2.5% HSA at 60 minutes' incubation.

The above results indicate that for IFN-β, PPF is a better stabilizer than HSA. In addition, tests for biological activity of representative formulated proteins at pH 3 revealed that the IFN-β was biologically active.

III. Low pH Mannitol Formulations

Stabilizers can be used to stabilize recombinant beta-interferon formulations at a low pH of 2-4 if the pH is maintained at 2-4 and lyophilized at pH 2-4. If the pH is raised above 4, however, the carbohydrate stabilizers such as mannitol will not act to solubilize the lipophilic protein. Only the protein stabilizers such as HSA will solubilize the protein as the pH is raised to physiological pH.

Conclusion

The process and compositions of the present invention as described herein yield a lipophilic protein preparation which is of relatively high purity, with residual SDS levels of less than about 10 p.p.m. and which may be formulated into therapeutically acceptable preparations in a non-toxic, inert, physiologically compatible carrier medium for clinical and therapeutic uses. The principal advantage of the instant invention lies in the reduction of SDS levels in the protein preparation (which can potentiate hepatic toxicity in some patients) to about 2-20 p.p.m., preferably to less than about 10 p.p.m., and more preferably to about 2-6 p.p.m., which are therapeutically acceptable. Although the preferred embodiment described relates to β-HIFN specifically, the purification methods of the instant invention can be used to purify other proteins with similar lipophilic characteristics to β-HIFN.

The foregoing description of the preferred aspects of the instant invention has been presented for purposes of illustration and description. It is not intended to be exhaustive or to limit the invention to the precise form disclosed, and obviously many modifications and variations are possible in light of the above teaching. The particular embodiments were chosen and described to explain best the principles of the invention and its practical application thereby to enable others skilled in the art to utilize best the invention in various embodiments and with various modifications as are suited to the particular use contemplated.

CLAIMS:

1. A stable pharmaceutical composition comprising a therapeutically effective amount of a biologically active recombinant β-HIFN dissolved in a non-toxic, inert, therapeutically compatible aqueous-based carrier medium at a pH of 2 to 4 comprising a stabilizer for the protein.

2. A composition according to claim 1 wherein the stabilizer is human plasma protein fraction and is present in an amount of 0.1 to 5% (w/v) or the stabilizer is human serum albumin and is present in a concentration range of about 0.5 to 10% (w/v).

3. A composition according to claim 1 wherein the stabilizer is a mixture of human serum albumin and dextrose and each are present in amounts of 1.25% (w/v).

4. A composition according to any one of claims 1 to 3 wherein the effective amount of β-HIFN is in the range of 0.1 to 1 mg β-HIFN per ml of said carrier.

5. A process for recovering a β-HIFN from a host transformed to produce it wherein the cell wall of the host is disrupted and the protein in the disruptate is isolated and purified, comprising the steps of:

(a) adjusting the pH of the medium in which the β-HIFN is contained to about 2 to 4;

(b) adding to the β-HIFN medium a stabilizer for the β-HIFN which has been

previously adjusted to a pH of about 2 to 4; and

(c) lyophilizing the resulting composition at about pH 2 to 4.

6. The process of claim 5 wherein the stabilizer is mannitol.

7. A process for recovering a β-HIFN from a host transformed to produce it wherein the cell wall of the host is disrupted and the protein in the disruptate is isolated and purified, comprising the steps of:

(a) adjusting the pH of the medium in which the β-HIFN is contained to about 2 to 4;

(b) adding to the β-HIFN medium a protein stabilizer for the β-HIFN protein which has been previously adjusted to a pH of about 2 to 4; and

(c) raising the pH of the resulting composition to 6.8-7.8.

8. A process for recovering a β-HIFN from a host transformed to produce it wherein the cell wall of the host is disrupted and the β-HIFN is isolated and purified, comprising:

(a) combining the β-HIFN with a protein stabilizer and adjusting the pH of the combination to about 2 to 4; and

(b) raising the pH of the resulting composition to 6.8 to 7.8;

the β-HIFN optionally being desalted at a pH of 9.2-11 by chromatography just prior to step (a).

9. A process for stabilising a biologically active β-HIFN comprising dissolving the β-HIFN in a non-toxic, inert, therapeutically compatible aqueous-based carrier medium at a pH of 2 to 4 comprising a stabilizer for the protein.

10. The use of a biologically active β-HIFN which has been stabilized by a process as defined in claim 9 in preparing a medicament.

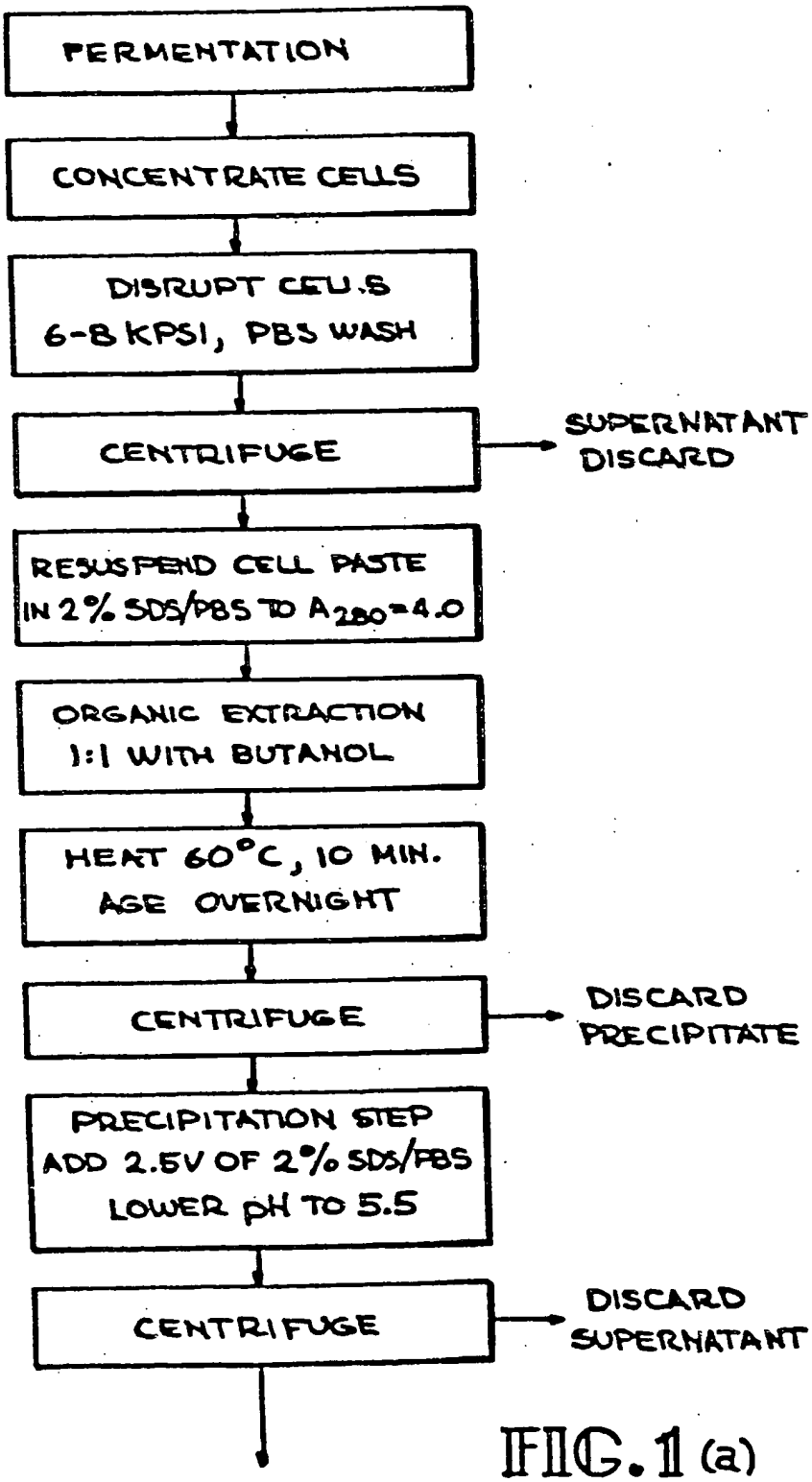
FERMENTATION
CONCENTRATE CELLS
DISRUPT CELLS
6-8 KPSI, PBS WASH
CENTRIFUGE
SUPERNATANT
DISCARD
RESUSPEND CELL PASTE
IN 2% SDS/PBS TO A_{280}=4.0
ORGANIC EXTRACTION
1:1 WITH BUTANOL
HEAT 60°C, 10 MIN.
AGE OVERNIGHT
CENTRIFUGE
DISCARD
PRECIPITATE
PRECIPITATION STEP
ADD 2.5V OF 2% SDS/PBS
LOWER pH TO 5.5
CENTRIFUGE
DISCARD
SUPERNATANT

FIG. 1 (a)

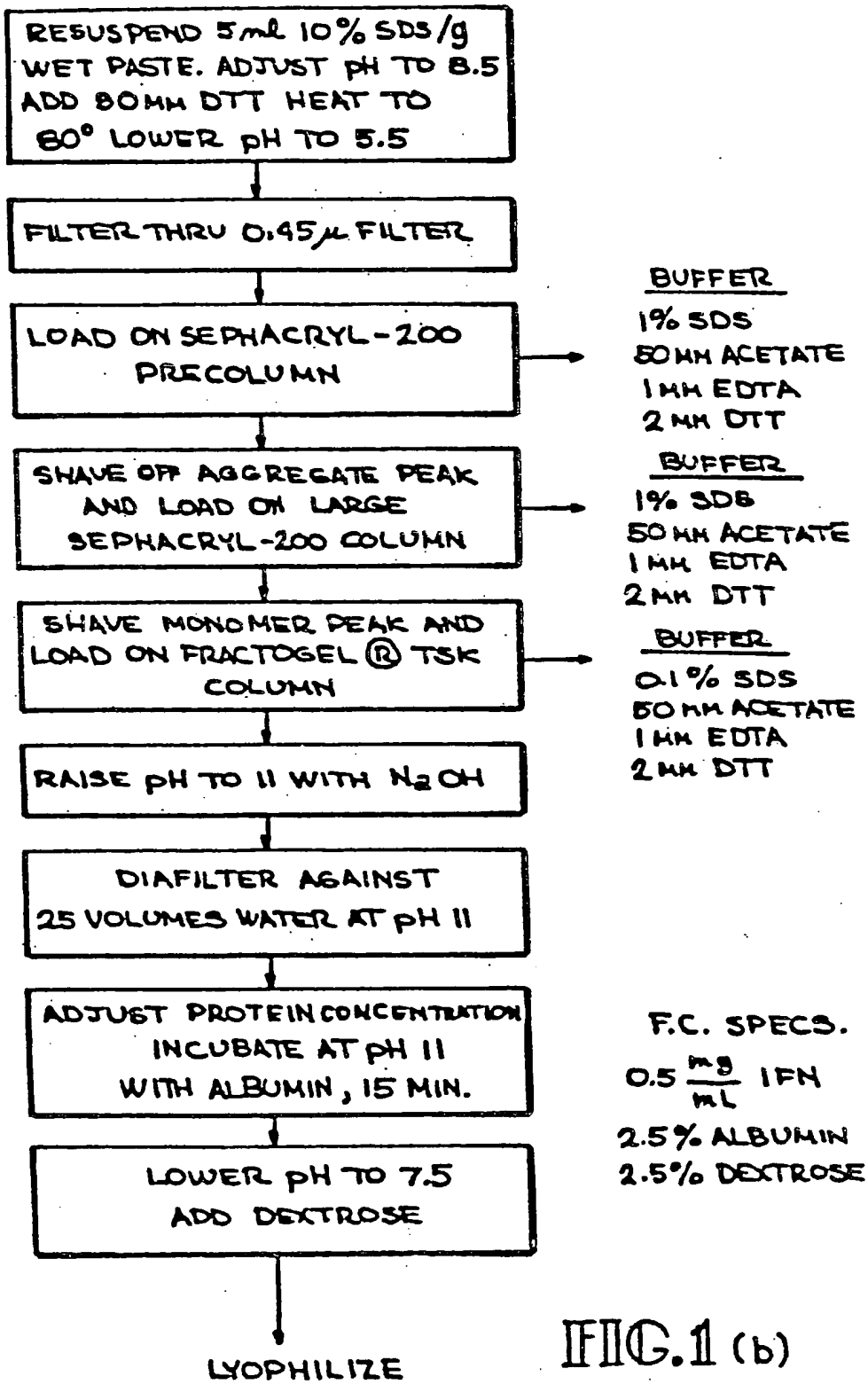
RESUSPEND 5ml 10% SDS/g WET PASTE. ADJUST pH TO 8.5 ADD 80MM DTT HEAT TO 80° LOWER pH TO 5.5
FILTER THRU 0.45μ FILTER
LOAD ON SEPHACRYL-200 PRECOLUMN
BUFFER
1% SDS
50 MM ACETATE
1 MM EDTA
2 MM DTT
SHAVE OFF AGGREGATE PEAK AND LOAD ON LARGE SEPHACRYL-200 COLUMN
BUFFER
1% SDS
50 MM ACETATE
1 MM EDTA
2 MM DTT
SHAVE MONOMER PEAK AND LOAD ON FRACTOGEL ® TSK COLUMN
BUFFER
0.1% SDS
50 MM ACETATE
1 MM EDTA
2 MM DTT
RAISE pH TO 11 WITH N_2OH
DIAFILTER AGAINST 25 VOLUMES WATER AT pH 11
ADJUST PROTEIN CONCENTRATION INCUBATE AT pH 11 WITH ALBUMIN, 15 MIN.
F.C. SPECS.
0.5 $\frac{mg}{mL}$ IFN
2.5% ALBUMIN
2.5% DEXTROSE
LOWER pH TO 7.5 ADD DEXTROSE
LYOPHILIZE

FIG. 1 (b)

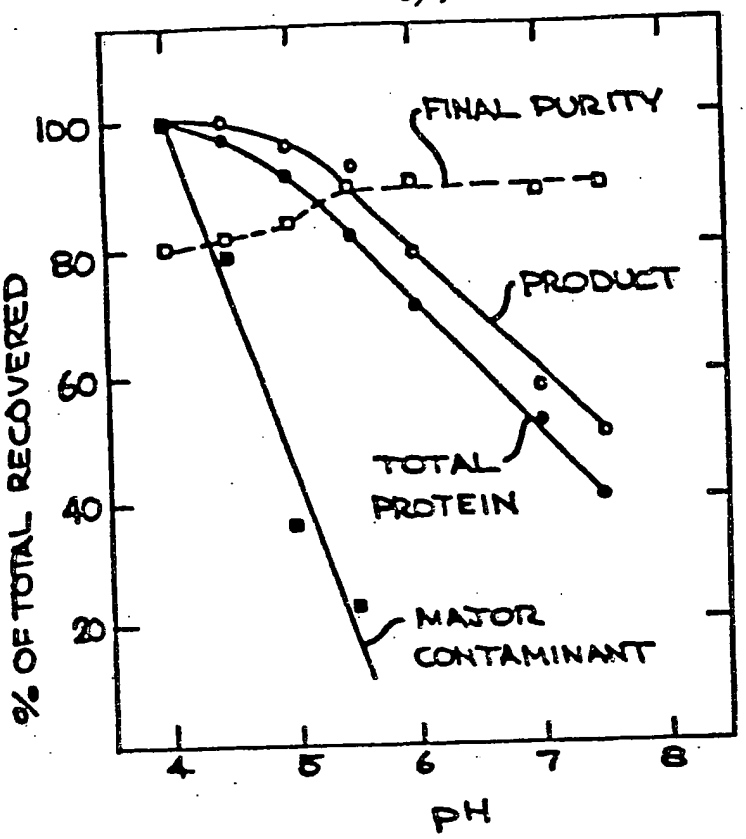
FINAL PURITY
PRODUCT
TOTAL PROTEIN
MAJOR CONTAMINANT
% OF TOTAL RECOVERED
100
80
60
40
20
4
5
6
7
8
pH

FIG. 2

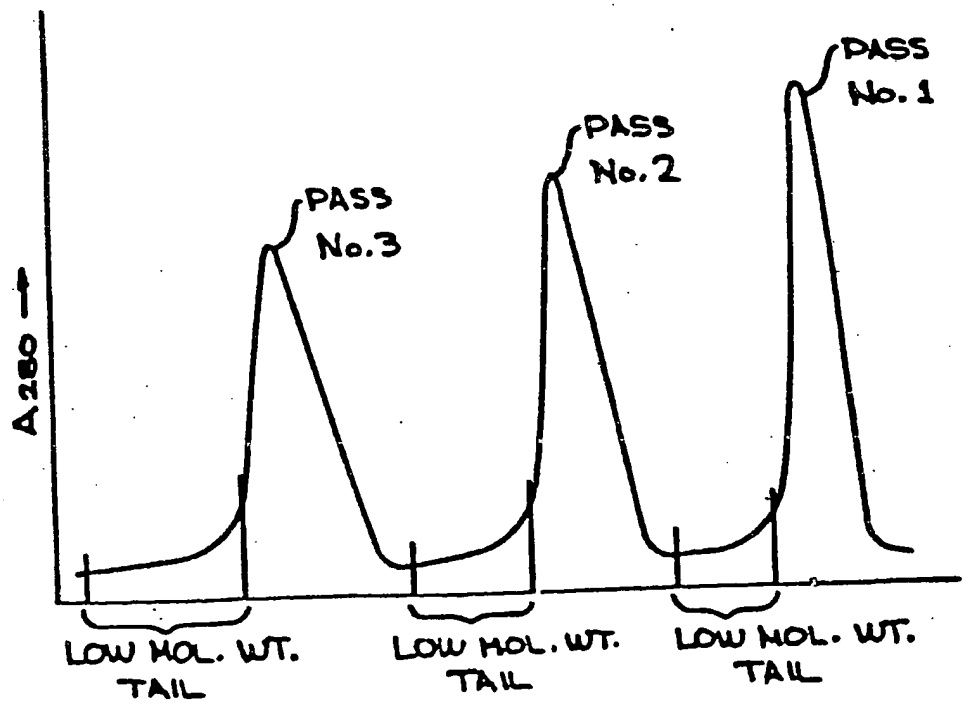
PASS No. 1
PASS No. 2
PASS No. 3
A280
LOW MOL. WT. TAIL
LOW MOL. WT. TAIL
LOW MOL. WT. TAIL

FIG. 3

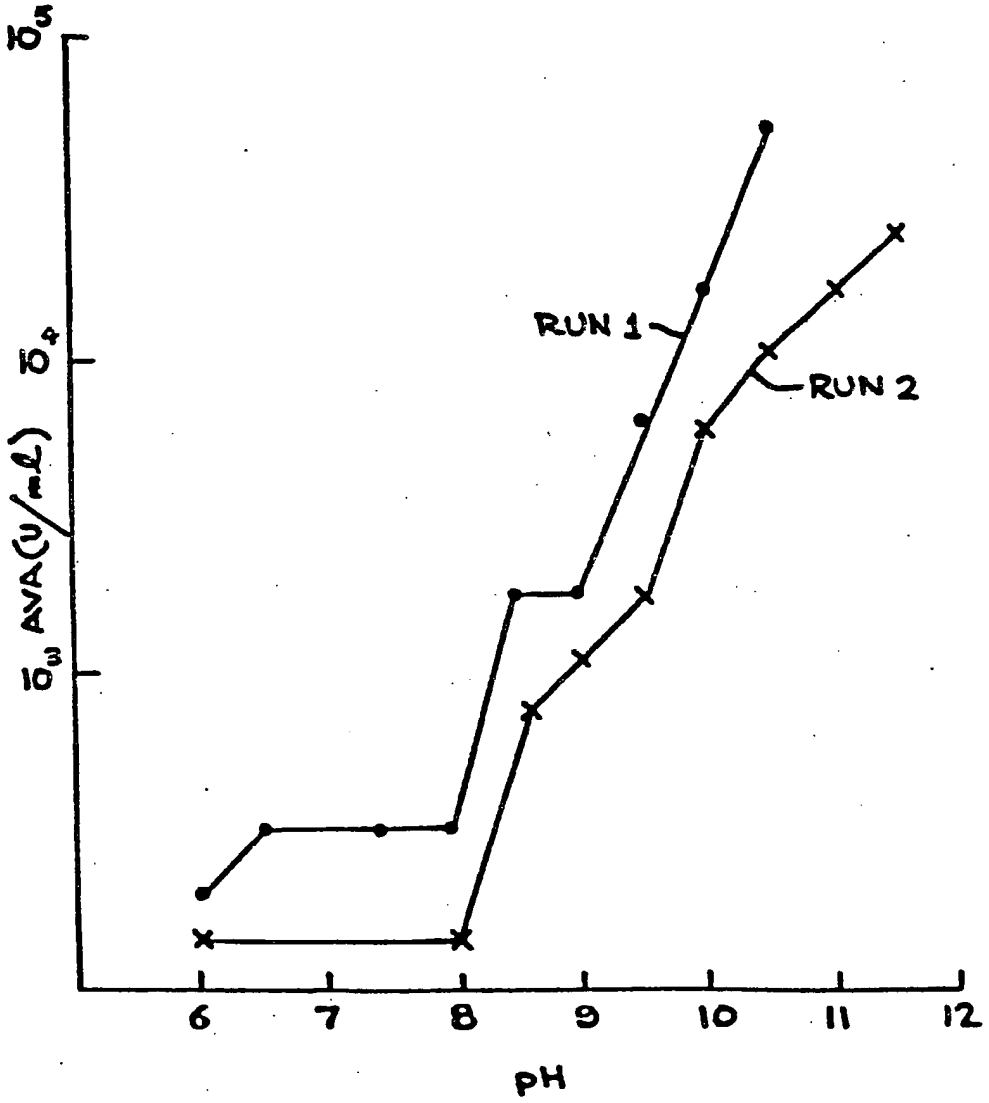

FIG. 4

Fermentation	
Cell concentration	
Cell membrane disruption	distilled water
Diafiltration	5 mM EDTA
Redisruption	2 mM EDTA 1% octanol (v/v)
Sucrose suspension	15-35% sucrose (w/w)
Centrifugation	10,000-20,000 x g
Solubilization	5% SDS, phosphate buffered saline
Centrifugation	25,000-35,000 x g
Reduction	5% SDS, 50 mM DTT, 2 mM EDTA pH 8.5 at 50°C for 20 minutes
Sephacryl S-200-A column	50 mM acetate, pH 5.5, 0.1% SDS, 1 mM EDTA
Oxidation	Iodosobenzoic acid (IBA) 1:1.6 molar protein:IBA, 0.1% SDS, 10 mM phosphate, pH 7.8, 1 mM EDTA

FIG. 5(a)

Concentration	pH 5.5
Diafiltration	0.1% SDS, 1 mM EDTA, 50 mM acetate pH 5.5
Filtration	pH $\leq$ 3, 0.45 µm filter
Preparative RP-HPLC	Vydac® C-4 bonded phase silica gel, 2 propanol/acetic acid
Dilution	1:14 into 50 mM acetate, pH 5.5, 1.0% SDS, 1 mM EDTA
Concentration	
Diafiltration	50 mM acetate pH 5.5, 0.1% SDS, 1 MM EDTA
Sephacryl S-200-B Column	50 mM acetate pH 5.5, 0.1% SDS, 1 mM EDTA
Desalting	G25 Sephadex column at pH 9.2-11
pH Adjustment	Separately adjust pH of protein and HSA or PPF to 2-4
Formulation	2.5% HSA or PPF (w/v), pH 2-4
Incubation	15 minutes
pH Adjustment	pH 7.5
Filtration	
Lyophilization	

FIG. 5(b)

Fermentation	
4M Urea Batch Extraction	
2-Butanol Extraction	
S-200 Column	1% SDS, 50 mM acetate 2 mM DTT, 1 mM EDTA, pH 5.5
G-25 Column	0.1% SDS, 50 mM acetate, 1 mM EDTA, pH 5.5
Oxidation	Iodosobenzoic acid
G-25 Column	0.1% SDS, 50 mM acetate, 1 mM EDTA, pH 5.5
RP-HPLC	Acetic acid, 2-propanol
Dilution/Diafiltration	1% SDS, 50 mM acetate, 1mM EDTA, pH 5.5
S-200 Column	1% SDS, 50 mM acetate, 1 mM EDTA, pH 5.5
Ultrafiltration	2 mM sodium phosphate, pH 7.5
Desalting	G25 column
Formulation	Adjust pH of HSA or PPF and protein separately to 2-4; mix
Incubation	15 minutes
pH Adjustment	pH 7.5
Lyophilization	

FIG.6